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THE NATURE OF HAEMOGLOBIN IN THE RED BLOOD CORPUSCLE

A PART OF A DISSERTATION SUBMITTED
TO THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

1938

By
GORDON ALBERT ADAMS

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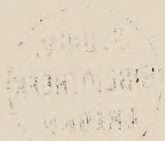
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LXXXV. THE NATURE OF HAEMOGLOBIN IN THE RED BLOOD CORPUSCLE

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IN a previous communication [Adams *et al.* 1934] it was shown that the ultra-violet band at 4100 Å. which is characteristic of haemoglobin and its immediate derivatives is not found in unlaked blood. This observation was made originally on corpuscle suspensions of horse blood, pure horse haemoglobin solutions and a series of derivatives made from purified horse haemoglobin (Hb). The observation has been extended to several species and confirmed in all cases. The blood specimens examined were goose, turtle, rabbit, horse and human.

Since this fundamental difference has been found, numerous attempts have been made to find out the nature of the Hb inside the corpuscle. Obviously, there is a difference between the Hb inside and outside the corpuscle. It may be a chemical or a physical difference due to different spectral conditions presented by a Hb solution and a corpuscle suspension of Hb. There is a possibility that the absence of the great u.v. band from the corpuscle suspensions might be due to the masking effects of the blood serum, the corpuscle wall, material mixed with Hb in the cell or material combined with Hb but removed either by laking. As to the serum it has been shown by Lewis [1917] that the u.v. absorption spectrum of blood serum has two peaks at 2800 and 2400 Å. respectively. It represents several superimposed curves and does not resemble that of a pure compound, being due chiefly to protein. Furthermore, it was noted that the great u.v. band of Hb exists in laked blood as well as in aqueous solutions of crystalline Hb. The question as to whether the corpuscles may be a factor can be eliminated by the fact that blood laked either by ether or by freezing and thawing and containing the corpuscle "ghosts" still displays the band in the ultra-violet. Removal of the "ghosts" by centrifuging does not effect the absorption band.

In regard to the presence of cell stroma masking the absorption band, it is evident that it is not a factor since the spectrum of the laked blood containing the empty corpuscles does not differ from that of an aqueous solution of oxy-haemoglobin.

Gibbs *et al.* [1931] call attention to the fact that in a system containing suspended matter capable of absorbing light, the total loss of light at any given wavelength is the sum of two factors, that due to selective absorption and that due to scattering. Two solutions of the same substance which contain different amounts of suspended matter yield absorption curves having the same general form but differing in intensity. This is the case using corpuscle suspensions and laked samples containing the "ghosts". In the case of the corpuscle suspension there is more suspended matter and consequently more scattering. The two absorption spectra should be the same in form but not in intensity. It has been shown that there is not only a change in intensity due to scattering but also a change in form as shown by the absence of the main band at 4100 Å. If the scattering due to the suspended corpuscles were responsible for the absence of the main band, then it would follow that the small band at 2750 Å. would be

lacking also. It is a law of optics that the loss of light due to scattering is greater toward the short wavelengths. However, the band at 2750 Å. appears in the corpuscle suspensions although diminished in its intensity compared with the laked blood spectra.

EXPERIMENTAL

In an attempt to get more direct evidence on the effect of scattering, some experiments were planned in which the volume of the corpuscle was altered and the effect on the u.v. spectrum recorded.

In order to change the volume of the corpuscles they were placed in solutions of different osmotic strengths. The normal, 0.9% saline, was regarded as the standard and others referred to it. Several other strengths varying from 0.5% saline to 1.4% were used. The blood cells were allowed to stand in contact with these solutions for 20 min. before the spectrogram was taken. Below 0.7% the experiments failed because of high cell fragility and resulting haemolysis.

The spectrum of the corpuscles in 0.7% saline shows a slight tendency towards a small band in the general region of 4100 Å.; but this is explained on the basis that there is always a slight degree of haemolysis in the hypotonic solutions and the small amount of Hb which "leaks" out is no doubt responsible for the small absorption at 4100 Å. From theoretical considerations a marked increase in absorption was expected. As a matter of fact, there was little increase in the absorption, showing that the increase in the size of the corpuscle had but little effect on the absorption intensity.

On the other hand, in the 1.2% saline the cells decrease in volume to about one-half their original size and consequently the light-scattering effect should be lessened; as a matter of observation the general absorption is slightly less, but here again the difference is not marked, so it may be concluded that the size of the particle is not more than a 2-4% factor in light absorption in the 4100 Å. region.

The next experiment was an attempt to establish artificial conditions which would approach the nature of the red blood cell suspension. This was done by precipitating Hb in a finely divided state, by adding gum arabic to the Hb solution and then sufficient dilute HCl to bring the Hb to its isoelectric point. This gave a very fine suspension of Hb which did not settle out on standing and was suitable for spectrographic analysis. The spectrum of this preparation was compared with that of a 10% Hb solution. The two spectra are so similar that the effect of precipitation in fine particles does not appear to make any marked difference. Actually the scattering effect of the particles does not amount to more than 5%, an amount which is not regarded as significant in this particular type of work.

Increased Hb concentrations do not affect the nature of the spectrum, which seems to be the same over the range 0.5-10%.

It is concluded on the basis of these experiments that the physical state of the Hb in the corpuscle is not sufficient to explain the lack of the characteristic u.v. band at 4100-4200 Å. It probably does not account for more than 5% of the difference in the absorption.

Since it may be concluded that physical differences between Hb inside and outside the corpuscle are inadequate to account for the lack of the characteristic band when Hb is in the corpuscle, attempts were made to find possible chemical combinations between Hb and cell constituents which would have spectra similar to Hb in the corpuscle. The fact that ether laking produces such marked changes points to the possibility that the cell constituents might be lipid in nature. However, the fact that laking brought about by the physical processes of freezing and thawing, which do not remove any of the constituents, still gives

the same spectrum as the ether-laked blood indicates that the presence of the band is independent of any ether-soluble substance which is removed by the laking process. It does not exclude the possibility that laking breaks down a lipid-Hb combination which lacks a characteristic band at 4100 Å.

Very little is known concerning the nature of Hb in the cell. It is not even known definitely whether it exists as a simple solution in a protein envelope or in a loose chemical union with stroma or some other cell constituent. No direct evidence has been brought to show that the Hb is combined with either the stroma protein, lipid fraction or any other cellular constituents.

In view of the lack of data in this regard attempts were made to combine Hb with some of the corpuscle constituents. The first one used was stroma protein or "stromatin". The stromatin was prepared by the method of Jorpes [1932] with some modification. The first preparations which were made contained approximately 10% Hb as determined by the iron content. The spectrogram of this substance shows a relatively flat curve in the region of 4100–4200 Å. Since the substance contained 10% Hb and yet showed no absorption in the 4100–4200 Å. region, it suggested a combination between the stromatin and Hb. The stromatin was rendered lipid-free by extraction three times with a 1 : 1 ether-alcohol mixture. The spectrum was again taken, and did not vary essentially from the previous one, showing that the lipid fraction is not concerned in the spectrogram.

Pure samples of stromatin were prepared, the only impurity encountered being methaemoglobin. Its presence could be quite easily detected spectroscopically. The stromatin was also rendered lipid-free by alcohol-ether extraction. The u.v. spectrum of this pure stromatin was used as standard for subsequent experiments. The material was prepared for spectrographic examination as follows.

To 2 ml. of the pure stromatin in alkaline solution (100 mg. in 25 ml. $N/10$ NaOH) there were added 10 ml. of 1% Hb solution. The pH of the solution was 9.8. The spectrogram was taken after about 5 min. The spectrogram is a typical haemoglobin one with the specific absorption at 4100–4200 Å. Apparently there is no immediate combination between stromatin and Hb. In the next experiment the same mixture was made, but it was heated at 37° for 30 min. The pH of this solution was 10.1. It changed from a clear reddish colour to a slightly opalescent yellowish red solution. The typical Hb band at 4100 Å. disappears completely and the general appearance of the absorption curve is similar to that of stromatin. It appears that heating of stromatin and Hb for even a period of 30 min. causes a combination of the two substances to give a new compound which has a spectrum very similar to that of stromatin. Heating the stromatin and Hb for 1 hr. instead of 30 min. does not affect the spectrum except to cause less general absorption. As a control experiment the effect of heating haemoglobin with the same amount of alkali as used to dissolve the stromatin was tried. This treatment did not affect the spectrum of the Hb.

A solution of stromatin was made up to a pH of 10.1 and heated for 1 hr. at 37° in order to find out if heating causes any change in its spectrum. There are no changes in the specific form of the spectrum curve, although the general absorption is slightly greater. Since it was possible to identify in the lipid fraction extracted from the crude stromatin both cholesterol and lecithin, attempts were made to combine these substances with Hb. This experiment might seem unnecessary since the entire crude lipid fraction did not show any signs of combination with Hb, but the possibility that the substance in pure form might react differently could not be disregarded.

The method employed was to add a few mg. of pure cholesterol to a drop of olive oil and 1 drop of $N/10$ NaOH. This mixture was then added to 10 ml. of a 0.5% Hb solution, shaken up thoroughly and heated for a period of 30 min. at 37° . At the end of the heating period, the spectrogram was taken. The spectrum curve is typical for Hb showing a distinct absorption band at 4100–4200 Å. Longer

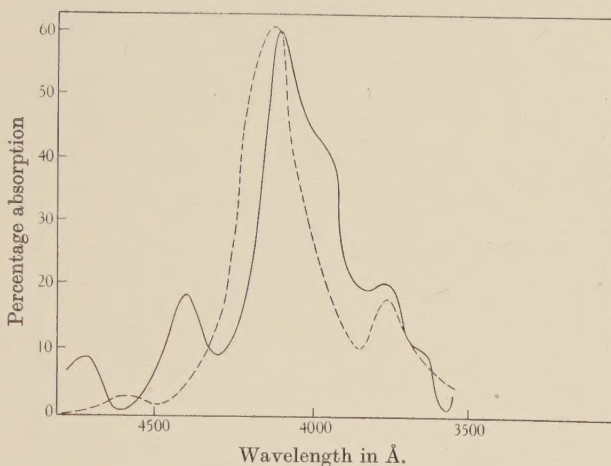


Fig. 1. Haemoglobin in finely precipitated state —. Haemoglobin in 10% solution ---.

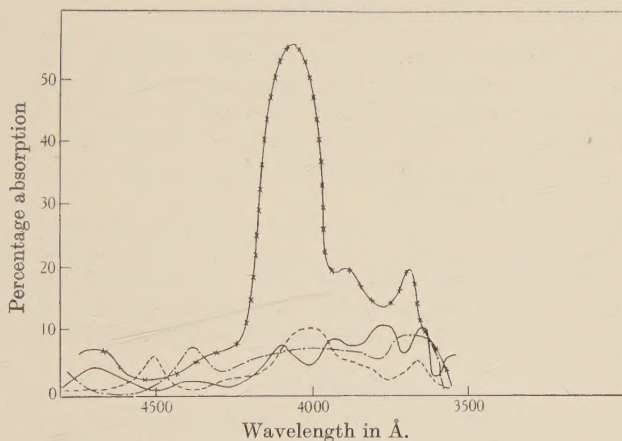


Fig. 2. Stromatin —. Stromatin (lipoid-free) ---. Stromatin-haemoglobin (no heating) $\times$ — $\times$. Stromatin-haemoglobin (heated 1 hr.) ----.

heating periods (up to 3 hr.) gave approximately the same result. The same experiment was tried with lecithin. The results again do not indicate any combination between Hb and lecithin that can be detected spectrographically.

Experimental evidence has been adduced by Litarczek [1933] to show that glutathione added to a Hb solution in the presence of an alkali causes the Hb spectrum bands in the visible region to become decreased or entirely to disappear. Litarczek's method of adding crystalline glutathione to a solution of Hb and heating to 35° was utilized for various periods, usually 30 min., 1 and $1\frac{1}{2}$ hr. The proportions used were 10 mg. glutathione to 20 ml. of 0.5% Hb with a drop

of $N/10$ NaOH added. The spectrum curve does not show any disappearance of the band in the u.v. and appears to be typical for Hb.

Attempts have been made to find out how the stromatin combines with the Hb molecule. When stromatin and alkaline haematin were heated together in the same way as the stromatin and Hb, no combination could be detected by

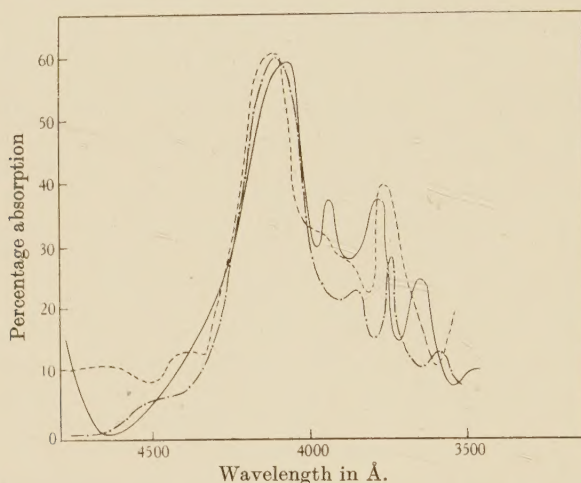


Fig. 3. Haemoglobin + lecithin (heated 1 hr.) ——. Haemoglobin + cholesterol (heated 1 hr.) ----. Haemoglobin + glutathione (heated 1 hr.) — · —.

changes in the spectrum. Reduced alkaline haematin and stromatin gave a substance with the spectrum of a haemochromogen with the typical 2-banded spectrum in the visible region and a well-defined band in the u.v. at 4000–4100 Å. Alkaline haematoporphyrin and stromatin have likewise shown no signs of combination. The stromatin therefore may be presumed to combine with the globin portion of the Hb molecule.

Isolation of the compound of stromatin and Hb has presented considerable difficulty. The compound appears to be quite labile and is readily broken down by a wide variety of reagents, e.g. many of the common haemolytic agents. Salting out experiments have brought down stromatin and Hb. Isoelectric precipitation has broken down the combination as shown by the presence of the spectrum of Hb.

SUMMARY

Blood corpuscle suspensions do not exhibit an ultra-violet absorption band in the region of 4000–4100 Å. *In vitro* experiments have shown that corpuscle “stromatin” and haemoglobin can be combined to give an ultra-violet spectrum similar to that of haemoglobin in the corpuscle. It is postulated that such a combination exists in the corpuscle.

The compound is a labile one and appears to be a union between stromatin and the globin fraction of the haemoglobin molecule.

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